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Detection of Protozoan Parasites and Microsporidia in Irrigation Waters Used for Crop Production

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ABSTRACT

The occurrence of human pathogenic parasites in irrigation waters used for food crops traditionally eaten raw was investigated. The polymerase chain reaction was used to detect human pathogenic microsporidia in irrigation waters from the United States and several Central American countries. In addition, the occurrence of both *Cryptosporidium* oocysts and *Giardia* cysts was determined by immunofluorescent techniques. Twenty-eight percent of the irrigation water samples tested positive for microsporidia, 60% tested positive for *Giardia* cysts, and 36% tested positive for *Cryptosporidium* oocysts. The average concentrations in samples from Central America containing *Giardia* cysts and *Cryptosporidium* oocysts were 559 cysts and 227 oocysts per 100 liters. In samples from the United States, averages of 25 *Giardia* cysts per 100 liters and <19 (average detection limit) *Cryptosporidium* oocysts per 100 liters were detected. Two of the samples that were positive for microsporidia were sequenced, and subsequent database homology comparisons allowed the presumptive identification of two human pathogenic species, *Encephalitozoon intestinalis* (94% homology) and *Pleistophora* spp. (89% homology). The presence of human pathogenic parasites in irrigation waters used in the production of crops traditionally consumed raw suggests that there may be a risk of infection to consumers who come in contact with or eat these products.

Foodborne illness affects approximately 76 million Americans each year, and the number of produce-related outbreaks is on the rise *(7, 16)*. The globalization of produce, an increase in the immunocompromised population, and increased consumption of fresh and organically grown produce may be causes of the heightened occurrence of produce-related outbreaks *(7)*. Sources of these produce-related outbreaks can be introduced during the growth, harvesting, processing, or distribution of produce *(22)*.

Irrigation waters may become contaminated either by the introduction of sewage or by runoff from nonpoint sources. Rain events may carry fecal contamination from agricultural, domestic, and wild animals (including birds) into canals, river waters, and wells that serve as sources of irrigation water *(7)*. The use of irrigation waters to mix insecticides and fungicides that are sprayed directly onto crops increases the risk of surface contamination by pathogenic microorganisms. The 1996 outbreak of cyclosporiasis may be one example of the transfer of protozoan pathogens from contaminated surface water to produce traditionally eaten raw. It has been suggested that contaminated surface water (found to contain cyclospora) that was sprayed onto raspberries prior to harvest may have been the cause of more than 1,400 cases of cyclosporiasis in Guatemala *(1, 12)*.

Two genera of waterborne protozoan pathogens, *Giardia* and *Cryptosporidium*, are transmitted via the fecal-oral route and are important causes of waterborne outbreaks of gastroenteritis. While no foodborne outbreaks due to contaminated irrigation water have been reported, other foodborne outbreaks have occurred *(21)*. It is likely that *Giardia* cysts and *Cryptosporidium* oocysts may occur in irrigation waters and survive on irrigated produce, since these pathogens may be transmitted by animals and are resistant to environmental conditions, and only a few cysts or oocysts may cause infection *(21)*.

For over half of the reported foodborne outbreaks, the etiological agent is unknown *(7)*. One group of emerging pathogens that has recently been implicated in waterborne disease is the microsporidia *(3)*. Microsporidia are parasites that cause infection in both vertebrates and invertebrates. Human microsporidia can potentially be transmitted through airborne, person-to-person, waterborne, and zoonotic modes *(2)*. The potential for zoonotic transmission of *Giardia* spp. and *Cryptosporidium* spp. is well documented, whereas evidence for the zoonotic transmission of microsporidia, important in human disease, is increasing *(4, 5, 19, 21)*. *Enterocytozoon bieneusi* has been detected in dogs, rabbits, and pigs *(5, 19)*. Potential reservoirs of *Encephalitozoon* spp. include rodents, rabbits, dogs, birds, pigs, monkeys, goats, and cattle *(2, 4)*.

Diseases caused by microsporidia are usually gastrointestinal, but infections of the respiratory, reproductive, muscle, excretory, and nervous systems have been reported *(26)*. Since the onset of the AIDS epidemic, *E. bieneusi* has been the microsporidian most commonly identified as the cause of chronic diarrhea and wasting in AIDS patients *(8)*.

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TABLE 1. *Locations and sources of sampled irrigation waters and types of produce affected by these waters*

Location	Irrigation water source	No. of locations	Produce impacted by sampled irrigation waters
Costa Rica	River	5	Cilantro, coffee, tomatoes, celery, lettuce, peppers
Mexico	Agricultural canal	11	Chili, cilantro, tomatoes, cucumbers, lettuce
Panama	River	5	Tomatoes, bananas, peppers, lettuce, potatoes, carrots, onions
	Lake	1	
United States	Agricultural canal	3	Lettuce, tomatoes

followed by *Encephalitozoon intestinalis*. Microsporidia have also been implicated in diseases affecting immunocompetent individuals *(2)*. *E. bieneusi* and *E. intestinalis* have been implicated in non-HIV-associated, self-limited diarrhea in several immunocompetent hosts *(2, 15, 18, 20)*. Recently, an outbreak of intestinal microsporidiosis was reported in France where contamination of the drinking water source was suspected to be the source of intestinal microsporidia detected in the feces of both immunocompromised and immunocompetent individuals *(3)*. One study has suggested that infection by *Encephalitozoon* sp. may be common in the immunocompetent population because of a high seroprevalence against *Encephalitozoon* spp. *(25)*. In addition, antibodies to *Encephalitozoon* sp. were detected in 21% of the households in a rural community in Mexico *(11)*. Because of asymptomatic or self-limited infection, microsporidiosis may go unrecognized in immunocompetent hosts.

Because of waterborne transmission of human pathogenic parasites, contamination of waters used for the irrigation of produce traditionally eaten raw may increase the risk of infection through the consumption of minimally processed fruits and vegetables. For these reasons, waters used to irrigate crops commonly consumed raw were collected from both Central America and the United States to determine the occurrence of human pathogenic microsporidia, *Giardia* cysts, and *Cryptosporidium* oocysts.

MATERIALS AND METHODS

Water sampling. Each water sample was collected from a different surface water source (a canal, a lake, or a river) that was used directly (with no prior treatment) for the irrigation of crops usually eaten raw. Table 1 lists the locations, the irrigation water sources, and the types of crops impacted by the irrigation waters tested. Water samples were processed according to the United States Environmental Protection Agency Information Collection Rule method *(24)*. Water samples (100 to 400 liters) were filtered through polypropylene wound filters by the sampling protocols described in the Information Collection Rule *(24)*. After collection, the filters were placed in separate plastic bags and held in ice-filled coolers until they arrived at our laboratory at the University of Arizona.

Purification of parasites. The filters were processed within 96 h of their arrival. Each filter was cut lengthwise, and the filter fibers were separated into two portions. Each portion was placed into a 4-liter beaker containing 1.5 liters of eluting solution *(24)*. The fibers were hand-washed for 30 min to dislodge any particulates, including microsporidia spores, trapped within the filter matrix. The filters were wrung out by hand after washing, and the elution solution was concentrated by centrifugation at 2,000 × *g* for 10 min in a Beckman GS-6 swinging bucket rotor centrifuge (with no brake). The supernatant was aspirated off, and the filter sediment was resuspended in elution solution. This step was repeated until all of the sample sediment was pelleted into one tube. The supernatant was again discarded, and the pellet was resuspended in 20% formalin. All samples were then stored at 4°C until floatation purification.

Floatation purification. Pelleted water concentrates were further purified by the floatation purification protocol described for the Information Collection Rule method *(24)*. A portion of the purified sample was then subjected to DNA extraction for detection of microsporidia or immunoflourescent staining and microscopy for the detection of *Giardia* cysts and *Cryptosporidium* oocysts.

Immunoflourescent staining and microscopy. Immunoflourescent staining with Hydrofluor flourescent antibodies (Hydrofluor Combo Meridian Diagnostics, Inc., Cincinnati, Ohio) and the detection of *Giardia* cysts and *Cryptosporidium* oocysts were carried out according to the Information Collection Rule protocols *(24)*.

DNA extraction. Total DNA was extracted from the purified samples with QIAamp Tissue Kit reagents (Quiagen, Inc., Santa Clarita, Calif.) according to slightly modified protocols described by Dowd et al. *(10)*. No more than 100 μl of pelleted water concentrate was resuspended in 180 μl of buffer ATL by vortexing. Next, 20 μl of proteinase K (100 mg/ml) was added, and the sample was once again vortexed. The sample was incubated at 55°C for 4 h in a shaking water bath. Next, 200 μl of buffer AL was added, and the sample was vortexed thoroughly and incubated in a 70°C water bath for 10 min and then incubated for 10 min in a 98°C water bath. Next, the samples were once again vortexed and centrifuged at 16,000 × *g* for 2 min to pellet any solids. The supernatant was transferred to a clean microcentrifuge tube, and the pellet was discarded. Next, 210 μl of 100% ethanol was added to the sample, which was thoroughly vortexed for 1 min. The sample was added to QIAamp spin columns and centrifuged at 6,000 × *g* for 1 min. The membrane-captured DNA was subsequently washed twice (per the manufacturer's instructions) and eluted by adding 100 μl of molecular-grade water that had been preheated to 70°C to the column and incubating the columns in a hybridization incubator for 10 min at 70°C. The samples were centrifuged for 1 min at 16,000 × *g*. The eluted water was reapplied to the column and incubated once again to maximize the recovery of DNA from the sample. Finally, the column was centrifuged at 16,000 × *g*, and up to 80 μl of the supernatant was used for the 100-μl polymerase chain reaction (PCR) analysis.

PCR. The PCR primers used in this analysis have previously been described *(10)*. The forward primer (5′-CAC CAG GTT GAT TCT GCC TGA C-3′) and the reverse primer (5′-CCT CTC CGG AAC CAA ACC CTG-3′) amplify the small-subunit ribosomal DNA (SSU-rDNA) of human pathogenic microsporidia. These microsporidia species include *E. bieneusi*, *E. intestinalis*, *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, *Vittaforma cornea*, and *Pleistophora* sp., all of which produce ~300-bp am-

TABLE 2. *Locations of irrigation waters and percentages of water samples containing* Giardia *cysts,* Cryptosporidium *oocysts detected by immunofluorescent staining and microsporidia detected by PCR compared with the total number of water samples analyzed*

Location	No. of samples	Percentage of samples positive for:		
		Microsporidial DNA	*Giardia* cysts	*Cryptosporidium* oocysts
Costa Rica	5	40	60	60
Mexico	11	27	64	18
Panama	6	<1	50	67
All Central American locations	22	16	59	36
United States	3	67	67	<1
All locations	25	28	60	36

plicon sizes. The PCR conditions were *Taq* Gold–induced (Perkin-Elmer Corp., Norwalk, Conn.) hot start cycling conditions consisting of 10 min of denaturation at 95°C followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 20 s, and extension at 72°C for 40 s. A final extension step consisting of 5 min at 72°C was included. PCR products were analyzed by agarose gel electrophoresis and observed for bands within the 300-bp region. Bands that were sequenced were cut from this region and purified for sequencing analysis.

Sequencing. PCR products were purified from the gel with a QIAquick PCR purification kit (Quiagen) and resuspended in sterile water. The forward PCR primer was then used for dye termination PCR sequencing, which was performed at the University of Arizona's Laboratory of Molecular Systematics and Evolution Sequencing Facility (Tucson, Ariz.).

SSU-rDNA sequence analysis. For SSU-rDNA sequence analysis, the National Center for Biotechnology Information's World Wide Web site (http://www.ncbi.nlm.nih.gov) was searched with BLAST 2.0.

RESULTS

Twenty-five different irrigation water samples from Central America (Mexico, Costa Rica, and Panama) and the United States were analyzed for the presence of human pathogenic protozoan parasites, *Giardia* cysts, *Cryptosporidium* oocysts, and human pathogenic microsporidia. Table 2 shows the percentages of samples that contained *Giardia* cysts, *Cryptosporidium* oocysts, and human pathogenic microsporidial DNA. The concentration ranges of *Giardia* cysts and *Cryptosporidium* oocysts for all irrigation water samples analyzed are shown in Table 3. The average numbers of cysts and oocysts detected in positive samples for each country (Costa Rica, Panama, Mexico, and the United States) and the combined averages for samples collected from Central American locations and samples collected from the United States are also listed in Table 3.

Seven out of 25 irrigation water samples from all locations were positive for human pathogenic microsporidial DNA. Two of the seven positive microsporidia PCR products were sequenced, and subsequent database homology comparisons allowed the presumptive identification of two human pathogenic microsporidial species. One irrigation water sample collected in Mexico was found to contain DNA from *E. intestinalis.* The homology of the National Center for Biotechnology Information database's sequence for *E. intestinalis* to the submitted irrigation water sequence was 94%. The second submitted irrigation water sequence, for a sample collected in Costa Rica, had a low (89%) homology to the National Center for Biotechnology Information database's sequence for *Pleistophora* species.

DISCUSSION

Cryptosporidium oocysts, *Giardia* cysts, and human pathogenic microsporidial DNA were detected in irrigation waters used for crops that require little processing. Since

TABLE 3. *Concentration ranges for all analyzed samples and averages for samples that contained cysts and oocysts*

Protozoan parasite	Sampling location	Cysts or oocysts per 100 liters	
		Range[a]	Geometric mean
Giardia cysts	Costa Rica	<52.6–17,493	6,426
	Mexico	<5.7–8,945	227
	Panama	<18–1,800	693
	Central America	<5.7–17,493	559
	United States	<7.7–40	25
	All	<5.7–17,493	369
Cryptosporidium oocysts	Costa Rica	<52.6–333	150
	Mexico	<5.7–1,579	612
	Panama	<18–250	190
	Central America	<5.7–1,579	227
	United States	<7.7–<43	<19
	All	<5.7–1,579	227

these pathogens are present in irrigation waters, they may come in contact with and attach to crop surfaces. In a study conducted by Monge and Chinchilla *(17)*, the presence of *Cryptosporidium* oocysts on fresh vegetables was suspected to be due to the use of contaminated irrigation waters. Monge and Chinchilla *(17)* also noted a larger number of oocysts on the tested vegetables during the rainy season. Agricultural, urban, and industrial runoff most likely contributed to the increased oocyst concentrations observed in their study. The irrigation waters in their study and in many of the Central American irrigation waters analyzed in this study are from surface waters that receive industrial, urban, and agricultural wastewater. Irrigation water samples were collected from rivers and lakes in Costa Rica and Panama and from irrigation canals in Mexico and the United States. Higher concentrations of *Giardia* cysts and *Cryptosporidium* oocysts were detected in water samples collected in the Central American countries than in those collected in the United States. One explanation may be that the locations in Central America were more heavily impacted by human activity and farm, wild, and domestic animal activity than were locations in the United States. Moreover, since human pathogenic microsporidia, *Cryptosporidium* oocysts, and *Giardia* cysts are excreted in the feces (microsporidia, *Giardia* spp., and *Cryptosporidium* spp.) or the urine (microsporidia) of infected individuals (animals and humans), irrigation water sources impacted by agricultural, urban, and wildlife pollution are more likely to have increased occurrences and concentrations of these pathogens.

Because of the low infectious doses required to induce infection by *Giardia* spp. and *Cryptosporidium* spp., the concentrations observed for many of the sampled waters may pose a health risk to consumers if these pathogens are associated with irrigated products. The infectious doses are not known for human pathogenic microsporidial species, but 28% of the sampled waters contained their DNA. Furthermore, two of these positive DNA samples were presumptively identified as the human pathogenic species *E. intestinalis* and *Pleistophora* spp., which are known to cause disease in individuals with compromised immune systems *(2)*. *E. intestinalis* has been identified as the cause of traveler's diarrhea, and a high seroprevalence of *Encephalitozoon* spp. has been reported in immunocompetent persons *(18, 25)*.

The methods used for the detection of human pathogenic microsporidia detect the presence of only DNA sequences specific to the human pathogenic microsporidial species and therefore do not discriminate between viable and nonviable spores. No methods are currently available to determine the viability of microsporidial spores from environmental samples. The methods used for the detection of *Cryptosporidium* oocysts and *Giardia* cysts cannot assess viability either. Although viability was not assessed for any of the irrigation water samples, previous research suggests that these protozoan pathogens are capable of surviving conditions associated with surface waters and produce surfaces. Kucerova-Pospisilova *(14)* suggested that *Encephalitozoon* spores were resistant to environmental conditions characteristic of surface waters. Germination and infection of *Encephalitozoon* spores were not affected by a week of storage at 4, 22, or 33°C *(14)*. *Giardia* cysts and *Cryptosporidium* oocysts are also able to survive for long periods in surface waters *(6, 13, 21)*.

The occurrences and concentrations of the studied pathogens may have been underestimated due to the methods used in this study. Previously reported efficiencies for the detection of *Giardia* cysts and *Cryptosporidium* oocysts averaged 11 and 26% at the University of Arizona *(23)*. Moreover, recovery of microsporidia is about 4.8%, lower than recovery of the other protozoan parasites analyzed *(9)*. The low recovery of these protozoan parasites may signify that their occurrence is higher than indicated by the results presented.

The results of this study show that microsporidial spores, *Giardia* cysts, and *Cryptosporidium* oocysts may come in contact with irrigated crops because of their presence in waters used for irrigation. Moreover, these pathogens may remain viable under conditions characteristic of surface waters and vegetable crop surfaces. Since the infectious doses for *Giardia* cysts and *Cryptosporidium* oocysts are low (there are no data available for the infectious dose of microsporidial species), the presence of viable organisms in irrigation waters that come in contact with minimally processed crops may increase the risk of disease to consumers who come in contact with or consume these products.

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